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Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1

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Virulence in the opportunistic human pathogen *Pseudomonas aeruginosa* is controlled by cell density via diffusible signalling molecules ('autoinducers') of the *N*-acylhomoserine lactone (AHL) type. Two *Bacillus* sp. isolates (A23 and A24) with AHL-degrading activity were identified among a large collection of rhizosphere bacteria. From isolate A24 a gene was cloned which was similar to the *aiiA* gene, encoding an AHL lactonase in another *Bacillus* strain. Expression of the *aiiA* homologue from isolate A24 in *P. aeruginosa* PAO1 reduced the amount of the quorum sensing signal *N*-oxododecanoyl-L-homoserine lactone and completely prevented the accumulation of the second AHL signal, *N*-butyryl-L-homoserine lactone. This strongly reduced AHL content correlated with a markedly decreased expression and production of several virulence factors and cytotoxic compounds such as elastase, rhamnolipids, hydrogen cyanide and pyocyanin, and strongly reduced swarming. However, no effect was observed on flagellar swimming or on twitching motility, and *aiiA* expression did not affect bacterial adhesion to a polyvinylchloride surface. In conclusion, introduction of an AHL degradation gene into *P. aeruginosa* could block cell–cell communication and exoproduct formation, but failed to interfere with surface colonization.

Keywords: virulence, cell–cell signalling, quorum sensing, twitching motility

INTRODUCTION

Cell–cell communication is crucial for the virulence of the opportunistic human pathogen *Pseudomonas aeruginosa*, which controls the production of extracellular virulence factors and toxic secondary metabolites via a complex regulatory cascade involving two autoinduction systems (reviewed by Fuqua & Greenberg, 1998; Pesci & Iglewski, 1999; Williams *et al*., 2000). The autoinducer synthase LasI generates primarily the *N*-acylhomoserine lactone (AHL) signalling molecule *N*-oxododecanoyl-L-homoserine lactone (OdDHL), as well as minor amounts of other oxo-AHLs such as *N*-oxohexanoyl-L-homoserine lactone (OHHL) and *N*-oxooctanoyl-L-homoserine lactone (OOHL) (Pearson *et al*., 1994). When OdDHL accumulates in a bacterial population with increasing cell density, it will interact, at a critical threshold level, with the transcriptional regulator LasR. The activated LasR protein stimulates the expression of *lasI*, generating the first autoinduction loop. LasR also positively controls the expression of *rhlR*, encoding the transcriptional regulator of the second autoinduction system involving *N*-butyryl-L-homoserine lactone (BHL). This C_4 AHL is made by the RhlI protein, which also generates small amounts of *N*-

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Abbreviations: AHL, *N*-acylhomoserine lactone; BHL, *N*-butyryl-L-homoserine lactone; CTAB, cetyltrimethylammonium bromide; HHL, *N*-hexanoyl-L-homoserine lactone; OdDHL, *N*-oxododecanoyl-L-homoserine lactone; OHHL, *N*-oxohexanoyl-L-homoserine lactone; OOHL, *N*-oxooctanoyl-L-homoserine lactone.

The GenBank accession number for the *aiiA* nucleotide sequence is AF397400. The GenBank accession numbers for the nucleotide sequences of the 16S rRNA genes of strains A23 and A24 are AF397398 and AF397399.

hexanoyl-L-homoserine lactone (HHL) (Winson *et al.*, 1995). Above a critical concentration, BHL activates RhlR, which induces the transcription of *rhlI*, thereby creating the second autoinduction loop. The expression of many virulence genes in *P. aeruginosa* requires LasR and/or RhlR, activated by their respective autoinducer molecules (Whiteley *et al.*, 1999). Therefore, virulence factors are produced when the density of the bacterial population is sufficiently high; this induction mechanism is known as quorum sensing (Fuqua & Greenberg, 1998; Williams *et al.*, 2000).

Null mutations in the chromosomal *lasI* and *rhlI* genes abolish autoinducer biosynthesis and strongly reduce virulence gene expression in *P. aeruginosa* (Brint & Ohman, 1995; Ochsner & Reiser, 1995; Pearson *et al.*, 1997, 2000; Pessi & Haas, 2000). LasI- and/or RhlI-negative mutants of *P. aeruginosa* are less virulent than is the wild-type strain in several animal models (reviewed by Rumbaugh *et al.*, 2000). Interference with quorum-sensing-dependent gene regulation may therefore be a new strategy to control *P. aeruginosa* infections (Kline *et al.*, 1999; Leadbetter, 2001). Several potential target sites in this complex regulatory network can be envisaged. For instance, antagonists of low molecular mass which compete for the autoinducer-binding site on the regulatory proteins may act as quorum sensing blockers. Indeed, the ability of natural and synthetic AHL analogues to interfere with quorum sensing has been well documented in various Gram-negative bacteria (de Nys *et al.*, 1993; Givskov *et al.*, 1996; Kuo *et al.*, 1996; Passador *et al.*, 1996; McClean *et al.*, 1997; Pesci *et al.*, 1997; Zhu *et al.*, 1998; Kline *et al.*, 1999; Manefield *et al.*, 2000). Alternatively, it might be possible to block cell–cell signalling by preventing AHL signal biosynthesis (i.e. by targeting the autoinducer synthases). Finally, enzymic signal destruction (Dong *et al.*, 2001) is another approach, which was chosen in the present work. Here we report on the isolation of a *Bacillus* gene encoding autoinducer degrading activity and we show that heterologous expression of this gene in *P. aeruginosa* strongly reduces autoinducer accumulation, virulence gene expression and swarming motility.

METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Unless indicated otherwise, *P. aeruginosa* and *Escherichia coli* strains were grown at 37 °C with shaking at 180 r.p.m. in nutrient yeast broth (NYB) or on nutrient agar plates (NA) (Stanisich & Holloway, 1972). The incubation temperature for the *Bacillus* strains A23 and A24 was 30 °C. Antibiotics, when required, were added at the following concentrations: tetracycline, 25 μg ml^{-1} for *E. coli* and 125 μg ml^{-1} for *P. aeruginosa*; ampicillin, 100 μg ml^{-1} for *E. coli*; carbenicillin, 250 μg ml^{-1} for *P. aeruginosa*. Liquid cultures of *P. aeruginosa* containing pME6000 (vector control) or PCR-amplified *aiiA* gene on pME6863 were grown without tetracycline in experiments designed to measure *lasB* and *rhlA* expression or to quantify autoinducers, pyocyanin and hydrogen cyanide (HCN). To counterselect *E. coli* S17-1 in matings with *P. aeruginosa*, chloramphenicol was used at 10 μg ml^{-1}. Flagellar swimming was tested on NYB solidified with 0·3 % agar (Serva). Plates were dried briefly and inoculated with bacterial overnight cultures grown on NA with tetracycline (if appropriate), using a sterile toothpick. Plates were incubated at 30 °C for 24 h and the diameters of the swimming zones were measured from three parallel experiments. Swarming was tested on plates containing 0·5 % (w/v) Bacto agar (Oxoid) with 8 g nutrient broth l^{-1} (Oxoid) to which 5 g glucose l^{-1} was added (Rashid & Kornberg, 2000). Swarm plates were dried very briefly and inoculated with 10 μl of bacteria grown overnight in NYB with tetracycline. Incubation was carried out at 37 °C for 16 h. Twitching motility was assayed on Luria broth (LB) medium (Sambrook *et al.*, 1989) solidified with 1 % bacto agar (Oxoid). Plates (2–3 mm thick) were stab-inoculated with sterile toothpicks to the bottom of the Petri dish from overnight cultures grown on NA containing, if appropriate, tetracycline. Subsurface twitching was evaluated after a 24 h incubation at 37 °C by measuring diameters of the twitch zones. Experiments were performed in triplicate. Rhamnolipid production was tested on agar plates containing cetyltrimethylammonium bromide (CTAB) and methylene blue (Siegmund & Wagner, 1991). To determine the concentration of pyocyanin, *P. aeruginosa* strains were grown with aeration in 20 ml glycerol-alanine medium (Frank & DeMoss, 1959). For quantitative HCN determination, cells were grown with oxygen limitation in tightly closed 125 ml bottles containing 20 ml glycine minimal medium (Castric, 1975). To avoid bacterial clumping, Triton X-100 was added to the liquid growth media at a final concentration of 0·05 % in experiments designed for autoinducer extraction, and for β-galactosidase, HCN and pyocyanin measurements.

Bioassay for the detection of soil isolates interfering with AHL-dependent gene regulation. The biotest which was developed here to screen a large collection of soil isolates is based on the fact that *P. aeruginosa* PAO1 produces two AHL autoinducers (BHL and HHL) which can restore violacein production to the autoinducer-negative mutant *Chromobacterium violaceum* CV026 (McClean *et al.*, 1997). Strain CV026 was streaked as a homogeneous line on a low-nutrient medium containing 4 g tryptic soy agar and 17 g agar l^{-1} (Serva). After incubation at 24 °C for 6 h, 5 μl of an overnight culture of a test strain was deposited at a distance of 6–7 mm from the CV026 line. After further incubation at 24 °C for 18 h, 5 μl of an overnight PAO1 culture was spotted at 10 mm from the test strain and 6–7 mm from the CV026 line. Incubation at 24 °C was continued for another 2 days before purple pigment production was evaluated. Test strains which interfered with AHL-dependent signalling reduced the localized, PAO1-induced production of violacein by CV026.

Strain identification. The 16S rRNA genes of isolates A23 and A24 were PCR amplified (3 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 54 °C, 1 min at 72 °C; 7 min at 72 °C) from chromosomal DNA using the universal primers 16SUNI-L (5′-AGAGTTTGATCATGGCTCAG-3′) and 16SUNI-R (5′-GTGTGACGGGCGGTGTGTAC-3′). PCR products were separated by agarose gel electrophoresis, purified and used as templates in cycle sequencing as described below.

DNA manipulations and nucleotide sequencing. Small-scale preparations of plasmid DNA were carried out by the CTAB method (Del Sal *et al.*, 1988) and large-scale preparations were performed using Qiagen-Tips (Qiagen). Chromosomal DNA was extracted from *Bacillus* strains A23 and A24 as follows. Bacteria of an overnight culture grown in 100 ml NYB were harvested, washed with 20 ml TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8) and stored at −80 °C. Frozen cells were ground in liquid nitrogen with mortar and pestle, resuspended

Table 1. Strains and plasmids

Strain/plasmid	Relevant characteristics/genotype	Source/reference
Strain		
P. aeruginosa		
PAO1	Wild-type	Holloway (1955)
PT623	*pilA*::Tc^r	Köhler *et al.* (2000)
MT1508	*str-8006*, *flaC303 chr1055*::Tn*501*; Sm^r, Hg^r	M. Tsuda collection
Bacillus subtilis	Wild-type	Laboratory collection
Bacillus spp.		
A23	AHL-degrading soil isolate from Ghana	This work
A24	AHL-degrading soil isolate from Switzerland	This work
E. coli		
DH5α	F$^-$ *recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* Δ(*lacZYA–argF*) *U169* λ^- [ϕ80d*lacZ*ΔM15]	Sambrook *et al.* (1989)
S17-1	*thi pro hsdR recA*; chromosomal RP4; Tra$^+$; Sm/Sp^r	Simon *et al.* (1983)
Plasmid		
pECP60	Translational *rhlA′–′lacZ* fusion; Ap^r	Pesci *et al.* (1997)
pME6000	Broad-host-range cloning vector; Tc^r	Maurhofer *et al.* (1998)
pME6860	pUK21 carrying the *aiiA* gene of strain A24 under constitutive *plac* control; Km^r	This work
pME6863	pME6000 carrying the *aiiA* gene of strain A24 under constitutive *plac* control; Tc^r	This work
pUK21	Cloning vector; ColE1 replicon; Km^r	Vieira & Messing (1991)
pTS400	Translational *lasB′–′lacZ* fusion; Ap^r	Passador *et al.* (1993)

in 2 ml TE buffer and 0·25 ml 10 % (w/v) SDS, and incubated at 37 °C for 15 min. After addition of 0·5 ml 5 M $NaClO_4$, the solution was extracted several times with equal volumes of phenol/$CHCl_3$ (1:1, v/v) and $CHCl_3$. Nucleic acids were precipitated with 0·1 vol. 3 M sodium acetate (pH 5·2) and 2 vols ethanol, washed with 70 % (v/v) ethanol, dried under vacuum and dissolved in 0·3 ml TE buffer containing 0·1 mg DNase-free RNase. Restriction enzyme digestions, ligations and agarose gel electrophoresis were done by standard procedures (Sambrook *et al.*, 1989). DNA fragments and PCR products were purified from agarose gels using Geneclean II Kit (BIO 101) and High Pure PCR Purification Kit (Roche Molecular Biochemicals). Transformation of *E. coli* was done by electroporation (Farinha & Kropinski, 1990). Southern blotting of *Bacillus* sp. DNA with Hybond-N membranes (Amersham), random-primed DNA labelling of a 0·75 kb *Xho*I–*Kpn*I *aiiA* fragment from pME6860 with digoxigenin-11-dUTP (Roche Molecular Biochemicals), hybridization and detection were performed according to the protocols of the suppliers. The nucleotide sequence of the *aiiA* gene carried by pME6860 was determined on both strands with a dye terminator kit (Perkin-Elmer product no. 402080) and an ABI PRISM 373 sequencer. Comparison of nucleotide and deduced amino acid sequences was performed using the Genetics Computer Group (GCG) program GAP. The *aiiA* nucleotide sequence will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession number AF397400.

The nucleotide sequences of the 16S rRNA genes from A23 and A24 were determined on both strands with the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit and an AB3100 sequencer. Eight separate PCR reactions (25 cycles of 10 s at 96 °C, 5 s at 50 °C, 4 s at 60 °C) were performed with the following primers: 16SUNI-L; 16SRNAI-S (5′-CTACGGGAGGCAGCAGTGGGG-3′) together with 16SRNA1S (5′-CTACGGGAGGCAGCAGTGAGG-3′); 16SRNAII-S (5′-GTGTAGCGGTGAAATGCGTAG-3′) together with 16SRNA2-S (5′-GTGTAGGGGTAAAATCCG-TAG-3′); 16SRNAV-S (5′-CCCCACTGCTGCCTCCCG-TAG-3′); 16SRNAVI-S (5′-CTACGCATTTCACCGCTA-CAC-3′) together with 16SRNA6-S (5′-CTACGGATTTTA-CCCCTACAC-3′); 16SRNAVIII-S (5′-GCGCTCGTTGCG-GGACTTAACC-3′) together with 16SRNA8-S (5′-GCGCT-CGTTATGGCACTTAAGC-3′); 16SRNAIV-S (5′-GGTTA-AGTCCCGCAACGAGCGC-3′) together with 16SRNA4-S (5′-GCTTAAGTGCCATAACGAGCGC-3′); and 16SUNI-R. Sequences were analysed using the GCG program FASTA. The nucleotide sequences of the 16S rRNA genes of strains A23 and A24 will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession numbers AF397398 and AF397399.

Plasmid constructions. The *aiiA* gene was PCR amplified (3 min at 95 °C; 21 cycles of 1 min at 95 °C, 1 min at 50 °C, 2 min at 72 °C; 10 min at 72 °C) from chromosomal DNA of *Bacillus* sp. strain A24 using the primers aiiA-7 (5′-ACGT-CTCG**AGGA**TCCAT*ATGACAGTAAAGAAGCTT*) and aiiA-8 (5′-GCTGGTACCGTCGAC*TATATATATTCAGG-GAA*); the ribosome-binding site is in bold, restriction sites for *Xho*I and *Kpn*I are underlined, and nucleotides corresponding to the 5′ and 3′ ends of the *aiiA* gene from strain 240B1 (Dong *et al.*, 2000) are in italics. The PCR product was cleaved with *Xho*I and *Kpn*I and cloned into pUK21 between the *Xho*I and *Kpn*I sites to give pME6860. In this construct, which is able to replicate in *E. coli*, *aiiA* expression is driven from the vector's constitutive *lac* promoter. To express *aiiA* in *P. aeruginosa*, pME6863, a derivative of the broad-host-range vector pME-6000, was constructed as follows. First, pME6860 was cleaved with *Kpn*I, polished with T4 DNA polymerase, and cleaved with *Xho*I to generate a 0·8 kb fragment carrying *aiiA*. This fragment was ligated, via an *Eco*RI–*Xho*I linker (5′-GAA-TTCCCGGGGATCCGGTGATTGATTGAGCAAGCTT-ATCGATACCGTCGACCTCGAG), with pME6000, which had first been linearized with *Bam*HI, treated with T4 DNA polymerase, and cleaved with *Eco*RI. The resulting

construct pME6863 expresses *aiiA* from the vector's constitutive *lac* promoter and the presence of the linker sequence, which carries translation stop signals in all three reading frames (underlined), prevents the formation of a potential LacZ′–AiiA fusion protein.

AHL degradation assays. *In vivo* degradation assays were performed as follows. Microtitre plates containing 10 μM HHL (Fluka) in NYB were inoculated with the bacteria to be tested (*Bacillus* sp. or *E. coli*) and incubated at 30 °C with gentle shaking for 16 h. Aliquots of 2, 5 or 10 μl of the bacterial suspensions were then transferred to a second microtitre plate containing 200 μl solidified NA per well. Bacteria were killed during a 10 min UV irradiation from a transilluminator and the wells were inoculated with 5 μl from an overnight culture of the indicator strain *C. violaceum* CV026. Violacein formation was monitored after a 24 h incubation at 30 °C. In wells which had been inoculated with test strains having autoinducer-degrading activity, the remaining concentration of HHL was insufficient to induce violacein formation in CV026.

AHLs used for *in vitro* degradation assays or as standards on TLC plates were purchased from Fluka or synthesized according to Chhabra *et al*. (1993) and were kindly provided by Markus Beyeler. *In vitro* degradation assays were carried out either with cell-free culture supernatants or with crude cell extracts prepared from test strains. Cultures (300 ml) of *Bacillus* strains A23 and A24 were grown in NYB at 30 °C overnight. Cells were collected by centrifugation, resuspended in 3 ml of a buffer containing 0·1 M potassium phosphate pH 7·0, 10 mM $MgCl_2$, 1 mM DTT and 10 % (v/v) glycerol, and broken by sonication. Crude extracts were separated from cell debris by centrifugation. Degradation assays contained 100 μl of crude extract or 100 μl of a cell-free culture supernatant, respectively, and were carried out at 30 °C in 1 ml of the same buffer. AHLs were added at the following final concentrations: 200 μM BHL, 3 μM OdDHL and 2 μM HHL. The quantity of each autoinducer used in these assays was 100–200 times higher than the respective detection limit (McClean *et al*., 1997; Shaw *et al*., 1997). After incubation for 6 h, the reaction mixture was adjusted to pH 5·0 with HCl and extracted with an equal volume of dichloromethane. The extract was dried under vacuum. The presence or absence of autoinducers was tested by TLC as described below.

Autoinducer detection and quantification by TLC. For detection of AHLs produced by PAO1 carrying pME6000 or pME6863, respectively, 20 ml cell-free supernatants of NYB cultures containing 0·05 % Triton X-100 were adjusted to pH 5·0 prior to extraction with 3 vols dichloromethane in a separating funnel. The solvent phase was treated with anhydrous $MgSO_4$ to eliminate H_2O and evaporated to dryness using a rotary evaporator. Depending on the optical density of the bacterial culture, the total extract was concentrated as follows: 1000-fold for $OD_{600} < 0{\cdot}5$, 500-fold for OD_{600} 0·5–1·4, 100-fold for OD_{600} 1·5–2·1 and 50-fold for $OD_{600} > 2{\cdot}1$. Samples from PAO1/pME6863, which contained very low amounts of BHL (and HHL), were concentrated up to 2000-fold. The presence of AHLs in these extracts was tested by C_{18} reverse-phase TLC, developed with methanol/water (60:40, v/v) and revealed by the indicator strains *C. violaceum* CV026 (McClean *et al*., 1997) and *Agrobacterium tumefaciens* NT1(pZLR4) (Cha *et al*., 1998). The amounts of OdDHL, BHL and HHL were estimated by comparison with three different dilutions of the respective standards.

Assays for exoproducts and β-galactosidase activity. HCN production by *P. aeruginosa* was quantified as described previously (Voisard *et al*., 1989). Pyocyanin was extracted with chloroform from cell-free culture supernatants of *P. aeruginosa* grown in glycerol-alanine medium and assayed spectrophotometrically at 520 nm (Essar *et al*., 1990). β-Galactosidase specific activities were determined by the Miller method (Sambrook *et al*., 1989).

Batch adhesion experiments. Batch adhesion experiments were carried out by adapting a method described by Rijnaarts *et al*. (1993). Glass vials (volume 14 ml) were filled to the top with degassed 0·1 M PBS (containing, per litre, 4·93 g NaCl, 0·29 g KH_2PO_4 and 1·19 g K_2HPO_4; pH 7·2) and sealed with rubber stoppers without leaving a head space. The rubber stoppers were pierced by a plastic-coated wire piece to which a PVC patch as the test surface was attached. Bacterial strains were grown at 37 °C to mid-exponential phase, harvested by centrifugation, and washed three times with 0·1 M PBS. Concentrated cell suspensions (100–300 μl) were gently injected through the stopper into the vials in order to obtain a final concentration of 4×10^7 bacteria ml^{-1}. The vials were placed on a slanted rotating wheel (7 r.p.m.; amplitude 10 cm) to avoid sedimentation of bacteria and incubated at room temperature (21 ± 3 °C). After 2 h incubation, the vials were opened and 70 ml 0·1 M PBS was added to each vial using a syringe. The tip of the needle was placed a few millimetres above the bottom of the vial and the flow was applied at a rate of 45 ml min^{-1}. The flow was aimed away from the PVC patch to prevent detachment of bacteria due to shear stress. The excess liquid was allowed to flow out of the vials. This dilution procedure served to avoid the subsequent passage of the PVC patches through an interface between a dense bacterial suspension and air. It has been observed that such a passage can bias adhesion results because it leads to collection of bacteria that have accumulated in the liquid–air interface (Schäfer *et al*., 1998). The PVC patches were carefully removed from the vials to prevent dewetting as much as possible, placed on a microscope slide and covered immediately with a coverslip. They were observed under a light microscope (magnification ×1250; BX-60, Olympus Optical Co.) equipped with a digital camera (SenSys, Photometrics). The numbers of adhered cells were determined for six randomly chosen locations, corresponding to a total area of $3{\cdot}56 \times 10^4$ μm^2. Locations close to the edges of the surfaces were avoided, since they gave erratic results, possibly due to deviating hydraulic conditions during incubation. Each experiment was done in triplicate. Levels of adhesion were given as numbers of cells per square centimetre and calculated by averaging the values of the 18 adhesion locations obtained for the three PVC patches.

RESULTS

Identification of bacterial rhizosphere isolates with autoinducer-degrading activity

A collection of 1300 bacterial strains obtained from rhizosphere soil of diverse geographical locations worldwide was screened for interference with AHL-dependent gene regulation, using a bioassay described in Methods. Among 16 candidates found, two isolates, A23 (from Ghana) and A24 (from Switzerland), showed BHL degrading activity. These isolates were taxonomically characterized as *Bacillus* spp. in that their 16S rRNA genes were more than 99 % identical with those from members of the *Bacillus cereus* group. The remaining soil isolates excreted diffusible compounds interfering with violacein production and were not analysed further. Culture supernatants and crude extracts were

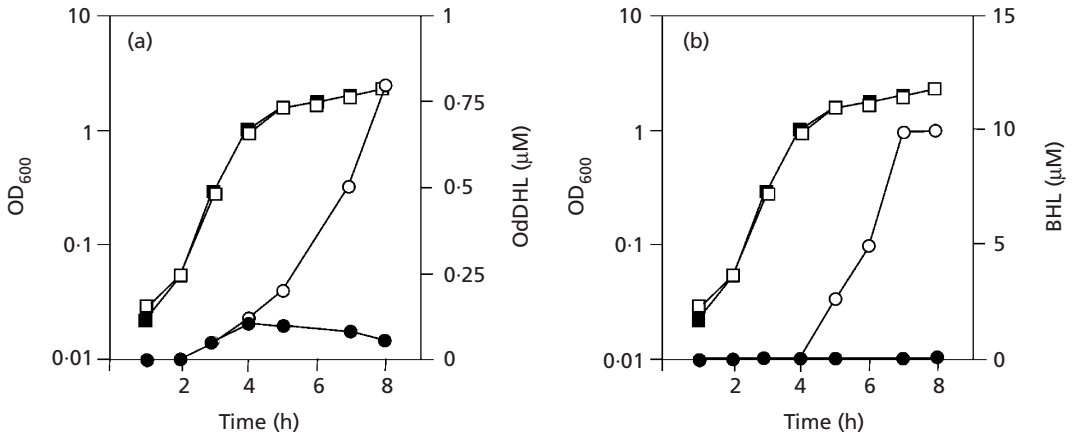

Fig. 1. Autoinducer accumulation (circles) during growth (squares) of *P. aeruginosa* PAO1/pME6000 (open symbols) and PAO1/pME6863 (filled symbols) in parallel batch cultures. At time 0, 50 ml flasks containing 20 ml NYB and Triton X-100 (0·05 %) were inoculated 1:100 and incubated at 37 °C with shaking for the time indicated. The OD_{600} of the cultures was measured and their supernatants were extracted and analysed by TLC for OdDHL (a) and BHL (b). The estimated error of this semiquantitative analysis was 20 % (see Methods). The detection limit for BHL in this assay was 0·25 μM.

prepared from strains A23 and A24 and incubated with 200 μM BHL for 6 h. The reaction mixtures were extracted and analysed by TLC for their BHL content. Whereas no degrading activity was detected in culture supernatants, cell extracts of both strains contained an activity degrading BHL to an undetectable level. HHL was also degraded under the same conditions (data not shown). By contrast, when the same experiments were performed with culture supernatants and crude extracts prepared from a *Bacillus subtilis* strain, no BHL-degrading activity was detected. These initial qualitative experiments show that the soil isolates A23 and A24 are able to degrade at least two different AHLs and that the respective enzymic activity is localized in the bacterial cytoplasm.

Cloning and sequence analysis of a gene from strain A24 responsible for autoinducer degradation

While this work was in progress, Dong *et al*. (2000) reported on a gene, *aiiA*, which encodes an AHL-degrading activity in *Bacillus* sp. 240B1. Based on the available sequence information, a potential *aiiA* homologue was PCR-amplified from strain A24 using the primers aiiA-7 (which adds a ribosome-binding site to the 5′ end of the gene) and aiiA-8 (see Methods), and cloned into the vector pUK21, to give plasmid pME6860. The nucleotide sequence of the pME6860 insert was determined on both strands. To ensure that the DNA sequence analysed was free of mutations potentially introduced during the PCR reaction, the fragment was PCR amplified, cloned and sequenced in two more independent experiments. The nucleotide sequences obtained were identical in all three experiments and found to be highly similar to the coding sequence of the *aiiA* gene from strain 240B1. The deduced amino acid sequence of the AiiA homologue from strain A24 differed from the sequence of strain 240B1 in the following positions: Val_{73}→Ile; Pro_{159}→Ser; Pro_{185}→Ser; Asn_{201}→Glu; Ser_{210}→Pro; Met_{225}→Ile; Arg_{241}→Lys. Autoinducer-degrading activity encoded by the cloned *aiiA* gene of strain A24 was confirmed with an *in vivo* degradation assay. *E. coli* DH5α expressing *aiiA* under the control of the constitutive *lac* promoter on pME6860 was able to degrade HHL whereas a control culture of DH5α was not.

To test whether a single or several *aiiA* genes were present in isolates A23 and A24, the 0·8 kb insert of pME6860 was used as a probe in a Southern blot against chromosomal DNA of A23 and A24. Hybridization under non-stringent conditions revealed single bands with six different restriction enzymes used. Both isolates gave identical patterns (data not shown). These results indicate that AHL-degrading activity is encoded in both bacterial isolates by a single *aiiA* gene.

Expression of *aiiA* in *P. aeruginosa* PAO1 decreases autoinducer concentrations

To study the effect of *aiiA* expression on autoinducer accumulation in *P. aeruginosa* PAO1, the *aiiA* gene of strain A24 was subcloned into the broad-host-range vector pME6000 under the control of the constitutive *lac* promoter. The resulting plasmid, pME6863, was mobilized, in parallel with the vector control (pME6000), from the *E. coli* donor strain S17-1 to *P. aeruginosa* PAO1. The influence of *aiiA* expression on the content of the two major autoinducers OdDHL and BHL of strain PAO1 was followed during growth. As illustrated in Fig. 1(a), *aiiA* expression severely reduced the content of OdDHL at high cell densities but had little effect on the low OdDHL concentration during exponential growth. The accumulation of the autoinducer BHL, which is produced later and at higher cell densities than is OdDHL, was completely prevented by *aiiA* expression (<0·25 μM; Fig. 1b). Minor products of LasI (OHHL and OOHL) and of RhlI (HHL) were also affected.

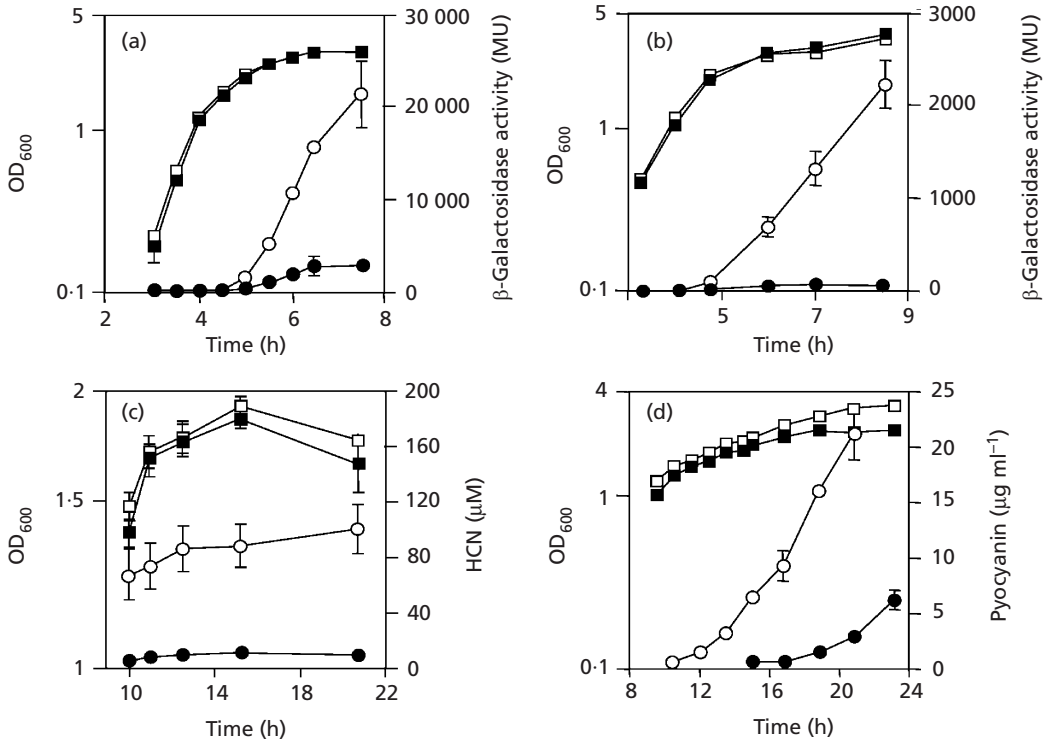

Fig. 2. Effect of *aiiA* expression on quorum-sensing-dependent phenotypes (circles) during growth (squares) of *P. aeruginosa* PAO1/pME6000 (open symbols) and PAO1/pME6863 (filled symbols). *β*-Galactosidase activity (in Miller units, MU) of a translational *lasB′–′lacZ* fusion carried by pTS400 (a) and of a translational *rhlA′–′lacZ* fusion carried by pECP60 (b), respectively, were determined from 20 ml NYB + Triton X-100 cultures inoculated 1:100 at time 0 and grown with shaking at 37 °C. For HCN determination (c), tightly capped 125 ml bottles containing 20 ml glycine minimal medium were inoculated to an OD_{600} of 0·002 and incubated at 37 °C with shaking. Samples (1 ml) were taken from the culture medium with a sterile syringe and assayed for their HCN content. For pyocyanin quantification (d), 50 ml flasks containing 20 ml glycerol-alanine medium were inoculated to an OD_{600} of 0·01 and incubated at 37 °C with shaking for the time indicated. The OD_{600} of the cultures was measured and their supernatants were extracted for pyocyanin quantification. The results shown in (a), (b), (c) and (d) represent means and standard deviations from three independent experiments.

Whereas at high cell densities the presence of *aiiA* strongly reduced the content of OHHL and OOHL, *aiiA* expression completely blocked the accumulation of HHL during the entire growth cycle (data not shown).

Autoinducer degradation reduces the production of several virulence factors

The effect of *aiiA* expression on quorum-sensing-dependent gene expression in PAO1 was evaluated. First, we measured the expression of the *lasB* gene, which encodes elastase, one of the virulence factors of *P. aeruginosa* (Blackwood *et al*., 1983). Elastase expression is controlled predominantly by the LasR/OdDHL quorum sensing system (Passador *et al*., 1993) and, to a lesser extent, also by the RhlR/BHL system (Brint & Ohman, 1995; Pearson *et al*., 1997). Expression of *lasB* was followed by monitoring *β*-galactosidase activity of a translational *lasB′–′lacZ* fusion on plasmid pTS400; in a PAO1/pME6863 background, *β*-galactosidase activity was significantly reduced compared to the activity measured in strain PAO1/pME6000 (Fig. 2a), indicating that *aiiA* strongly reduces the expression of elastase.

We next evaluated the effect of *aiiA* on rhamnolipids, which have both haemolytic and biosurfactant properties (Johnson & Boese-Marrazzo, 1980; Koch *et al*., 1989). The biosynthesis of rhamnolipids requires the *rhlAB*-encoded rhamnosyltransferase, whose expression is controlled mainly by the RhlR/BHL autoinduction system and, to a lesser extent, by LasR/OdDHL (Brint & Ohman, 1995; Pearson *et al*., 1997). Expression of a translational *rhlA′–′lacZ* fusion carried by plasmid pECP60 was severely reduced in PAO1 expressing *aiiA* (Fig. 2b). These results were confirmed by semi-quantitative rhamnolipid assays. Overnight cultures (10 μl) were spotted on agar plates containing CTAB and methylene blue and the radius of each clearing zone was measured after 48 h incubation. Rhamnolipid production in three independent experiments was significantly smaller in PAO1/pME6863 (radius of clearing 2·0 ± 0·1 mm) than in PAO1/pME6000 (radius 3·5 ± 0·1 mm).

aiiA expression also affected the production of HCN, which has been shown to be important for the virulence of *P. aeruginosa* towards *Caenorhabditis elegans*

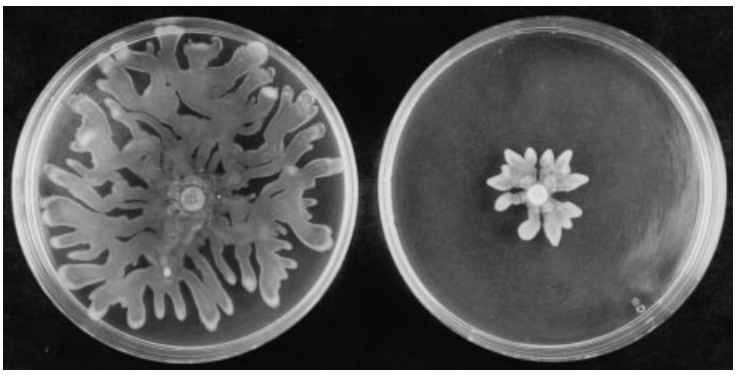

Fig. 3. Effect of *aiiA* expression on swarming motility of *P. aeruginosa* PAO1. Ten microlitres of overnight cultures of PAO1/pME6000 (left) and PAO1/pME6863 (right), respectively, were deposited on semisolid agar plates (see Methods) and incubated at 37 °C for 24 h.

(Gallagher & Manoil, 2001). The expression of the biosynthetic genes *hcnABC* requires both quorum sensing systems for maximal activity and is controlled additionally by the anaerobic activator ANR (Pessi & Haas, 2000). HCN was produced by strain PAO1/pME6863 in only very small amounts (about 10 μM), whereas concentrations of about 100 μM were measured in cultures of PAO1/pME6000 (Fig. 2c). Thus, HCN production is severely hampered in PAO1 expressing *aiiA*.

Similar results were also obtained for the production of the blue pigment pyocyanin, which, together with the siderophore ferripyochelin and a reducing agent such as NADH, catalyses hydroxyl radical formation and thus contributes to tissue injury (Britigan, 1993; Mahajan-Miklos *et al*., 1999). The expression of the pyocyanin biosynthetic genes strongly depends on quorum sensing and involves both autoinduction systems (Whiteley *et al*., 1999). Pyocyanin formation was followed during growth of PAO1/pME6863 and PAO1/pME6000, respectively. As shown in Fig. 2(d), *aiiA* expression dramatically delayed and reduced the formation of this toxic compound.

Effect of *aiiA* expression on motility of *P. aeruginosa*

Expression of *aiiA* in strain PAO1/pME6863 strongly reduced swarming motility on 0·5% agar medium containing the attractant glucose (Fig. 3). *P. aeruginosa* requires flagella, type IV pili and rhamnolipid production for swarming motility (Köhler *et al*., 2000). We therefore tested whether *aiiA* expression would affect surface translocation by swimming, which requires flagella, or twitching motility, which depends on type IV pili (Henrichsen, 1972; Bradley, 1980). On 0·3% agar plates, concentric rings with similar diameters (30 $\pm$1 mm) were formed by PAO1/pME6000 and PAO1/pME6863, indicating that swimming motility was not altered. In a control experiment using the *flaC* negative mutant MT1508, no swimming motility was observed. Similarly, the zones of subsurface twitching were indistinguishable for both strains (27$\pm$6 mm for PAO1/pME6000 and 24$\pm$2 mm for PAO1/pME6863) on twitch plates whereas no twitch zone (<1 mm) was formed by the pilus-negative (*pilA*) mutant PT623 used as a control. From these experiments we conclude that decreased swarming motility in the strain expressing *aiiA* (PAO1/pME6863) probably results from the strongly diminished rhamnolipid production.

aiiA expression does not reduce adhesion of *P. aeruginosa*

Flagella and type IV pili also promote bacterial adhesion to abiotic surfaces and, as a consequence, are involved in a first step of biofilm formation (O'Toole & Kolter, 1998). We tested whether *aiiA* expression affected adhesion of strain PAO1/pME6863 to a polyvinylchloride (PVC) surface (see Methods). Adhesion efficiency of this strain ($1{\cdot}83\pm0{\cdot}41\times10^6$ cells adhered cm^{-2}; mean$\pm$SD) was similar to that measured with PAO1 ($1{\cdot}71\pm0{\cdot}19\times10^6$ cells cm^{-2}) or PAO1 carrying the vector control pME6000 ($1{\cdot}74\pm0{\cdot}34\times10^6$ cells cm^{-2}). Adherence of the *pilA* mutant PT623, by contrast, was strongly reduced in this assay ($0{\cdot}61\pm0{\cdot}16\times10^6$ cells cm^{-2}).

DISCUSSION

Cell–cell signalling molecules of the AHL type are produced by many Gram-negative bacteria, where these small molecules are involved in the regulation of a vast variety of bacterial phenotypes (Swift *et al*., 1999). Since AHL-producing bacteria are ubiquitously present in natural environments, it can be expected that other organisms have evolved means to interfere with this type of communication, perhaps to defend themselves in their ecological niches. The seaweed *Delisea pulchra*, for example, produces several halogenated furanones, which are structurally related to AHLs (de Nys *et al*., 1993). Some furanones have been shown to inhibit AHL-mediated gene expression by displacing the AHL signal from its receptor protein (Givskov *et al*., 1996; Manefield *et al*., 1999, 2000). Another demonstrated mechanism of interference is signal destruction. AHL degradation was demonstrated first in the *Bacillus* soil isolate 240B1, from which a gene was cloned (*aiiA*), encoding an acyl homoserine lactonase (Dong *et al*., 2000, 2001). AHLs are also metabolized by a recently isolated bacterium, *Variovorax paradoxus*, which

hydrolyses the amide bond of these signalling molecules and utilizes them as the sole source of energy and nitrogen (Leadbetter & Greenberg, 2000).

In the present work, we have undertaken an independent search for bacteria which could interfere with the quorum sensing system of *P. aeruginosa*. Two *Bacillus* strains, A23 and A24, isolated from a Ghanaian and a Swiss soil, respectively, were able to degrade several AHLs. Despite the fact that the respective enzymic activity was localized in the bacterial cytoplasm, these strains were picked up in our screen. Apparently, effective intracellular degradation of the diffusible signal molecules HHL and BHL (produced by PAO1 in this assay) reduced the local signal concentration to an extent which resulted in a lower pigment production by the indicator strain.

We found that AHL-degrading activity was encoded in strain A24 by a gene whose DNA sequence was very similar to the previously described *aiiA* sequence (Dong *et al*., 2000), and a related gene was shown to be present also in strain A23. The AiiA lactonase is assumed to be a metallohydrolase, whose conserved histidine and aspartate residues in two different sequence motifs are characteristic of zinc-binding enzymes. These residues, which are entirely conserved in the deduced amino acid sequence of the *aiiA* homologue of strain A24, are crucial for AHL-degrading activity (Dong *et al*., 2000). Of the seven amino acid differences observed between strains A24 and 240B1, three changes concern proline residues, which could have an impact on the protein's secondary structure. It will therefore be interesting to compare the substrate specificities of the two proteins.

Heterologous expression of *aiiA* in *P. aeruginosa* PAO1 completely prevented the accumulation of the RhlI-generated autoinducers BHL and HHL throughout growth and severely reduced the concentrations of the oxo-AHLs produced by LasI at high cell densities, whereas low levels of OdDHL formed in the exponential-growth phase escaped AiiA action (Fig. 1). As the two autoinduction circuits form a regulatory cascade in which the Rhl system depends on a functional Las system, degradation of the oxo-AHLs by AiiA will reduce the synthesis of BHL and HHL. Thus, the absence of these two autoinducers throughout growth of strain PAO1 carrying pME6863 probably results from reduced synthesis as well as from AiiA-dependent degradation. However, we cannot exclude the possibility that AiiA might degrade non-oxo-AHLs more efficiently than oxo-AHLs.

Our finding that degradation of cell–cell signalling molecules in strain PAO1 expressing the A24 *aiiA* gene severely affected the expression and production of elastase, rhamnolipids, HCN and pyocyanin (Fig. 2) is in general agreement with the established fact that both AHLs are crucial for virulence gene expression in *P. aeruginosa* (Pesci & Iglewski, 1999). In the same vein, Dong *et al*. (2000, 2001) have shown that expression of *aiiA* from *Bacillus* sp. 240B1 in the plant pathogen *Erwinia carotovora* reduces autoinducer accumulation, decreases extracellular pectolytic enzyme activities and attenuates pathogenicity on several plant species. Our work opens up the possibility to test the impact of signal destruction on the pathogenicity of *P. aeruginosa in vivo*.

Even though *aiiA* expression strongly reduced AHL content in strain PAO1, we did not observe any significant effect on twitching motility and on adherence to PVC. This result was somewhat unexpected as twitching motility of *P. aeruginosa* has been reported to be under quorum sensing control (Glessner *et al*., 1999). Twitching motility depends on type IV pili (Bradley, 1980), which are also required for adhesion of *P. aeruginosa* to eukaryotic cell surfaces (Ramphal *et al*., 1984, 1991; Sato *et al*., 1988; Tang *et al*., 1995) and play a role in the early stages of biofilm formation on abiotic surfaces (O'Toole & Kolter, 1998). Although pilin mRNA levels were not regulated by quorum sensing in strain PAO1 (Pearson *et al*., 1997), surface piliation has been found to be strongly reduced in the BHL-negative (*rhlI*) mutant PDO100 (Glessner *et al*., 1999). We have shown here that twitching motility was not affected when BHL was degraded below the limit of detection in strain PAO1 expressing *aiiA* on pME6863 (Fig. 2b). This strongly suggests that a functional quorum sensing system is not required for this type of surface translocation. Twitching defects were recently found to develop spontaneously in *lasI* and *rhlI* mutants (Whitchurch *et al*., 2001) and it is therefore possible that a spontaneous mutation accounts for the reduced twitching motility in the *rhlI* mutant PDO100.

As illustrated by this study, enzymic AHL destruction by *aiiA* expression offers a possibility to examine the role of AHL-dependent quorum sensing without the need for mutant construction. AiiA-dependent signal destruction may therefore be a particularly useful tool to study the impact of quorum sensing in Gram-negative bacteria having multiple AHL regulatory circuits.

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